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Direct Development in the Ascidian *Molgula retortiformis* (Verrill, 1871)

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Abstract. The cellular features of the ascidian *Molgula retortiformis* (Verrill, 1871), a direct developing species, were investigated with the aid of transmission electron microscopy, histochemistry, and immunocytochemistry. Developmental comparisons between direct and indirect developing ascidians will further our understanding of how developmental processes evolve. *M. retortiformis* eggs are surrounded by a follicular envelope comprising a layer of outer follicle cells attached to an acellular chorion. The cytoplasm of *M. retortiformis* eggs contains large quantities of yolk and glycogen. Immediately after hatching, at day 2.5 of development, the cells constituting a juvenile exhibited similar ultrastructural features, except that the larger, deeper cells contained more yolk and glycogen than the epidermal cells. Differentiated muscle cells were absent in newly hatched *M. retortiformis* juveniles, and acetylcholinesterase (AChE) activity was not detected. Immunocytochemistry experiments using a vertebrate intermediate filament antibody (NN18) support the idea that the failure of newly hatched *M. retortiformis* juveniles to develop muscle cells may be due to the absence of a factor localized in the egg myoplasm. This paper concludes with a discussion of the "substrate hypothesis" and the evolution of ascidian direct development.

Introduction

Most ascidians produce eggs that develop into chordate larvae that swim for a brief time and subsequently metamorphose into adults. During metamorphosis the chordate features of a larva are selectively destroyed, and the adult morphology develops (Grave, 1935; Cloney, 1978, 1982). Ascidians that produce swimming larvae are termed indirect-developing species. In striking contrast to indirect-developing species, about a dozen species produce fertilized eggs that develop directly into juveniles, bypassing the development of a swimming larva (de Lacaze-Duthiers, 1874; Berrill, 1931; Jeffery and Swalla, 1990; Bates and Mallett, 1991a). Here I report on the cellular features of a direct-developing species, *Molgula retortiformis*.

N. J. Berrill (1931) wrote that *M. retortiformis* has a direct mode of development; however, he provided only one line drawing of a juvenile. His drawing shows a *M. retortiformis* juvenile without a tail, lacking a sensory vesicle, having partially extended epidermal ampullae, and containing a cluster of large, opaque cells, which he terms "tail phagocytes," in the posterior region. Aside from these general features, no information was given on the cellular features of eggs, embryos, and juveniles in this species. Although Berrill was not concerned primarily about the cellular features of direct-developing ascidians, he was among the first to recognize that comparisons between indirect and direct modes of ascidian development can provide valuable insights about chordate evolution. In his 1931 paper, Berrill suggested that direct development in ascidians evolved by the elimination of the larval sensory vesicle and larval tail structures. He argued that the development of a swimming tadpole larva capable of selecting a habitat would be unnecessary if the adult lived in a uniform habitat. This idea, which is termed the "substrate hypothesis," is based primarily on studies of *Molgula occulta*, a direct-developing species that inhabits the sand flats of Brittany. I reexamine Berrill's substrate hypothesis in the present study of *M. retortiformis*.

Interest in ascidian direct development was renewed when Whittaker (1979) reported that *Molgula arrenata* embryos, embryos exhibiting direct development, can express acetylcholinesterase despite the lack of tail development. AChE activity in a species with direct develop-

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ment suggested to Whittaker that AChE activity is a vestigial trait that has not been eliminated from an ancestral program responsible for larval muscle cell development. The present study tested the possibility that newly hatched *M. retortiformis* juveniles can express AChE activity. Just-hatched tadpoles from three indirect-developing species, *Halocynthia pyriformis, Boltenia echinata,* and *Ciona intestinalis,* were also tested for AChE activity.

Since the publication of Whittaker's exciting results in 1979, a number of studies on ascidian direct development have been reported, including those by Young *et al.* (1988), Jeffery and Swalla (1990, 1991, 1992), Bates and Mallett (1991a,b), Bates (1991), and others. In 1988, Young *et al.* were the first to report that *Molgula pacifica* is a direct developer. Many of the cellular features of *M. pacifica* development have been described (Bates and Mallett, 1991a,b; Bates, 1991, 1993). The postfertilization movements of the egg cytoplasm, termed ooplasmic segregation, and early cleavage patterns in *M. pacifica* were similar to those in eggs and embryos having indirect development. Although most features of early development were similar to those in indirect developers, ampulla development in *M. pacifica* juveniles was triggered *before* hatching (Bates and Mallett, 1991a; Bates, 1993, 1994) instead of after larval settlement (Cloney, 1978; Grosberg, 1981; Grosberg and Quinn, 1986).

The elimination of larval muscle cell development in direct-developing ascidians was recently studied in *Molgula oculata* (an indirect-developer) and *Molgula occulta* (a direct-developer), the same species studied by Berrill (1931). Results of these studies suggested that the lack of larval muscle cell development in *M. occulta* may be due to the absence of a protein that is recognized by a vertebrate intermediate filament antibody (NN18) localized in the myoplasm of *M. oculata* eggs (Swalla *et al.*, 1991). In the present study, I used *M. retortiformis* and an indirect-developing species, *Boltenia villosa,* to test the correlation between the antigen recognized by NN18 and AChE activity.

In summary, the threefold aim of the present study was (1) to examine the general cellular features of *M. retortiformis* eggs, embryos, and juveniles; (2) to determine if there is a correlation between AChE activity and a factor localized in the egg myoplasm that reacts with NN18 in *M. retortiformis* juveniles and *B. villosa* tadpoles; and (3) to test Berrill's substrate hypothesis by examining the habitats of *M. retortiformis* adults.

Materials and Methods

Collection of adults, eggs and sperm, and embryo cultures

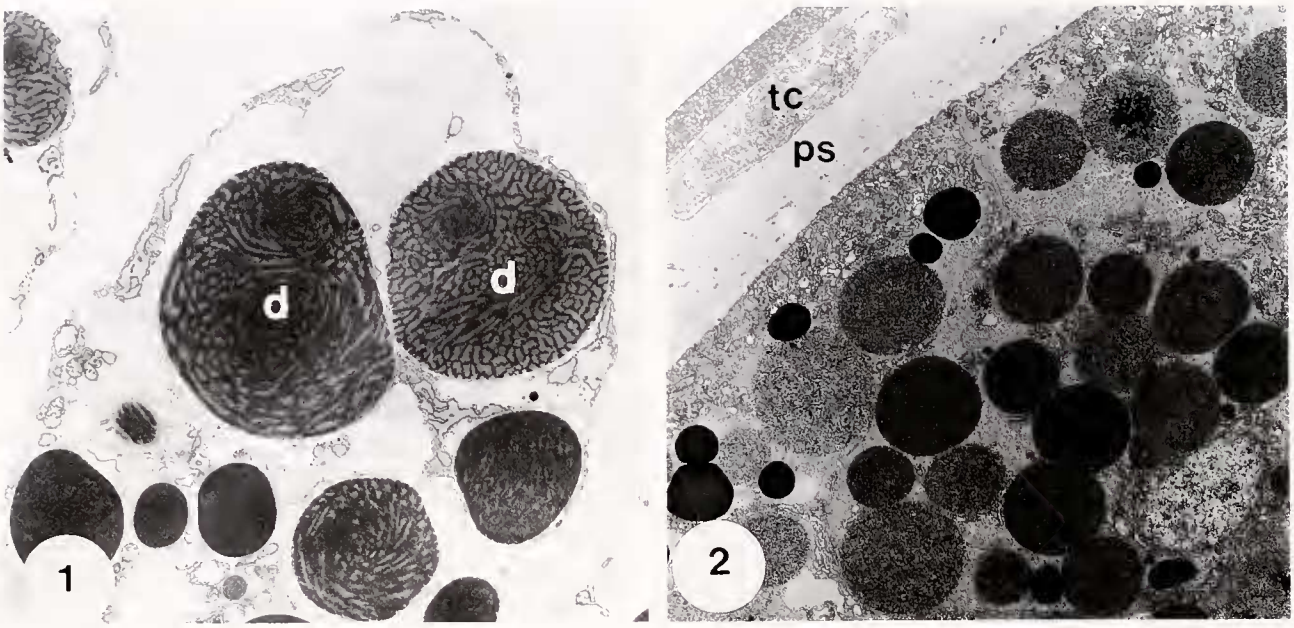
Molgula retortiformis, Halocynthia pyriformis, Boltenia echinata, and *Ciona intestinalis* adults were collected in the Bay of Fundy near Huntsman Marine Station, St. Andrews, New Brunswick, Canada. Collections were made with a dredge at depths ranging from 50 to 100 feet. *Boltenia villosa* adults were purchased from Westwind Sealab Supplies, Victoria, British Columbia. Adults were maintained in aquaria containing flowing seawater under conditions of constant light to prevent spawning. Testes and ovaries were removed from adults and placed in a Syracuse dish containing seawater; eggs and sperm were collected by using forceps to macerate the gonads. Van Name (1945) described *M. retortiformis* (Verrill, 1871). The testis on the left side of an adult was situated alongside the inner side of the lower branch of the intestinal loop and the left ovary was situated outside the intestinal loop along the upper branch of the intestinal loop. On the right side, the testis was situated ventral to the kidney and the ovary was situated along the dorsal border of the kidney. Fertilized eggs were obtained by mixing together eggs and sperm from two or more individuals in a Syracuse dish containing Millipore-filtered seawater. Eggs were inseminated for 10 min, washed with large volumes of seawater, and cultured at 11°C. Embryos were viewed at frequent intervals with an Olympus SZ stereomicroscope.

Transmission electron microscopy

Embryos and juveniles were prepared for light microscopy and transmission electron microscopy as previously described by Bates and Mallett (1991a). Specimens were fixed in 2% glutaraldehyde in 0.1 *M* sodium phosphate buffer, pH 7.4, for 30 min. After a wash in the same buffer, the specimens were immersed in 1% osmium tetroxide in the same buffer for 1 h. Specimens were dehydrated through a graded series of ethanol dilutions (10%–100%), then immersed in propylene oxide and gradually infiltrated with Spurr low-viscocity resin. Thick and thin sections were cut; the thick sections were stained with methylene blue and azure B, and the thin sections were immersed in uranyl acetate. The thin sections were viewed with a Phillips electron microscope at 80 kV. As a positive control, hatched *B. villosa* larvae were prepared for transmission electron microscopy along with hatched *M. retortiformis* juveniles. In every *B. villosa* preparation examined, sarcomeres were clearly evident within the tail muscle cells.

Acetylcholinesterase histochemistry

Day 2 *M. retortiformis* juveniles, *Boltenia echinata, Halocynthia pyriformis,* and *Ciona intestinalis* larvae were tested for acetylcholinesterase activity as previously described by Karnowski and Roots (1964), Whittaker (1973), and Bates and Jeffery (1987). Wholemount preparations were viewed with an Olympus microscope and photographed with Plus X film.



Figures 1 and 2. Transmission electron micrographs of a sectioned *Molgula retortiformis* follicle cell (1) and a sectioned *M. retortiformis* gastrula (2). The swirl patterns of follicle cell droplets (d) are evident in (1) and a test cell (tc) is seen within the perivitelline space (ps) in (2). × 3300 in (1) and in (2).

Immunocytochemistry

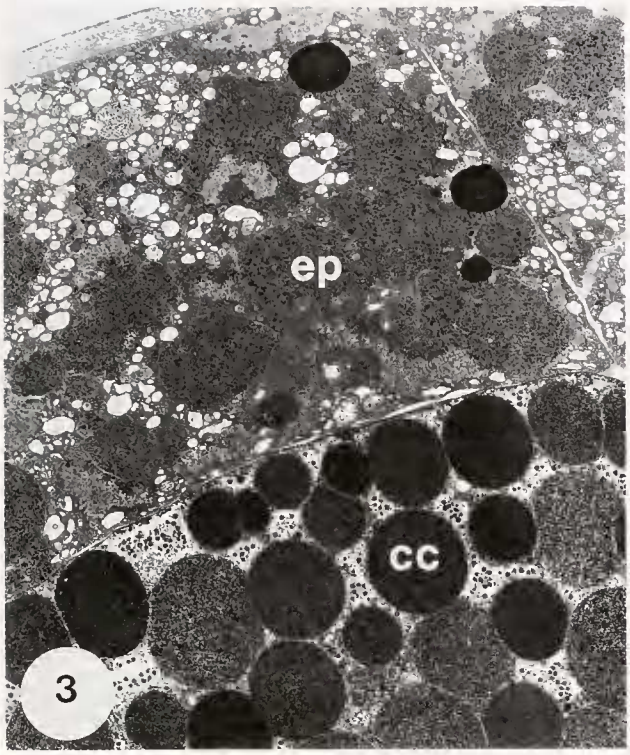
M. retortiformis and *B. villosa* eggs were prepared for immunocytochemistry, as previously described by Mita-Miyazawa *et al.* (1987). Eggs were immersed for 20 min in absolute methanol, and then for 20 min in cold absolute ethanol. Fixed eggs were infiltrated with 50% polyester wax (BDH Limited, Poole, England): absolute ethanol for 1 h at 40°C and then infiltrated with 100% polyester wax for 1 h at 40°C. Specimens were embedded in BEEM capsules, and 8-μm sections were cut from the blocks. Sections were mounted on gelatin-coated coverslips, de-waxed through a graded series of ethanol dilutions (100%; 90%; 80%; 70%; 50%; 30%), and rinsed in phosphate buffered saline (PBS). The specimens were incubated with a monoclonal antibody (1:25 dilution of NN18 from Sigma Chemicals) for 1 h at room temperature, washed with PBS, and incubated for 50 min in a 1:60 dilution of FITC-conjugated IgG (Sigma Chemical Company), as previously described by Swalla *et al.* (1991). The specimens were washed in PBS for 30 min, mounted in 80% glycerol dissolved in PBS, and viewed with an Olympus fluorescence microscope. Sections were photographed with Tri X film, ASA 400.

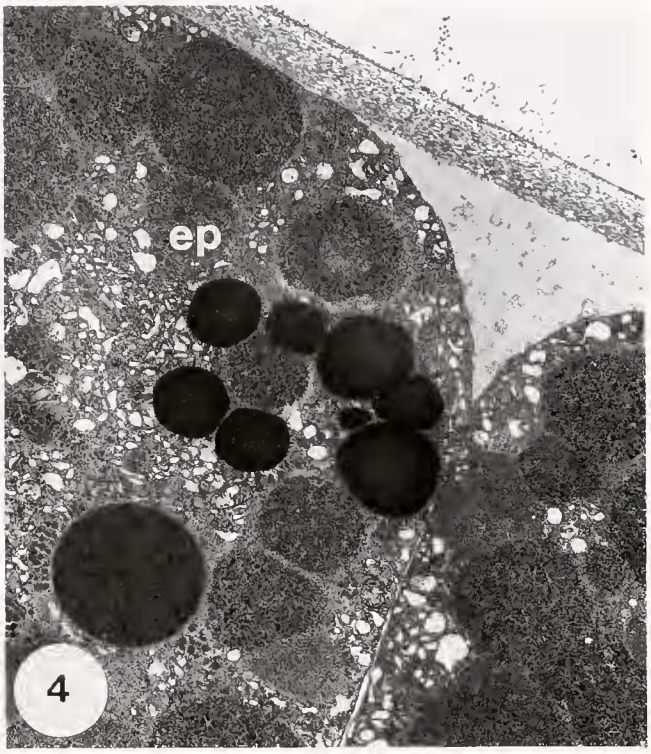
Results

A large population of *M. retortiformis* adults was discovered living on an underwater hill near Huntsman Marine Station at a depth of 50–100 feet. The animals were attached directly to rocks and lived close to several other ascidian species, *Boltenia ovifera, Molgula citrina, Ascidia callosa,* and *Halocynthia pyriformis.* The *M. retortiformis* adults collected from the underwater hill ranged from about 20 to 75 mm in diameter. Only a few specimens were collected from sand and gravel sites dredged near the underwater hill, suggesting that *M. retortiformis* adults prefer a hard substrate.

Maximum egg diameters (not including the surrounding follicular envelope) were 230–240 μm. The ultrastructural features of *M. retortiformis* follicle cells are shown in Figure 1. The cytoplasm of follicle cells contained droplets of various sizes, the contents of which display swirl patterns. Follicle cells are attached to an acellular chorion separated from the plasmalemma of the egg by a narrow perivitelline space. Cells within the perivitelline space, termed test cells, were observed in a few sections (Fig. 2).

The cytoplasm of *M. retortiformis* eggs contains large quantities of yolk and glycogen. After an egg was cross-fertilized, a thick coat of sticky adhesive material anchored it to the bottom of the glass culture dish. Fertilization triggered a rapid rearrangement of the egg cytoplasm, known as ooplasmic segregation. Opaque cytoplasm moved into one region of the egg and subsequently, just before first cleavage, formed a narrow belt of opaque cytoplasm in the equatorial region. Unlike the eggs of several other species, including *B. villosa,* the egg of *M. retortiformis* does not have colored pigment granules in its cortex. In some of the fertilized eggs, ooplasmic movements





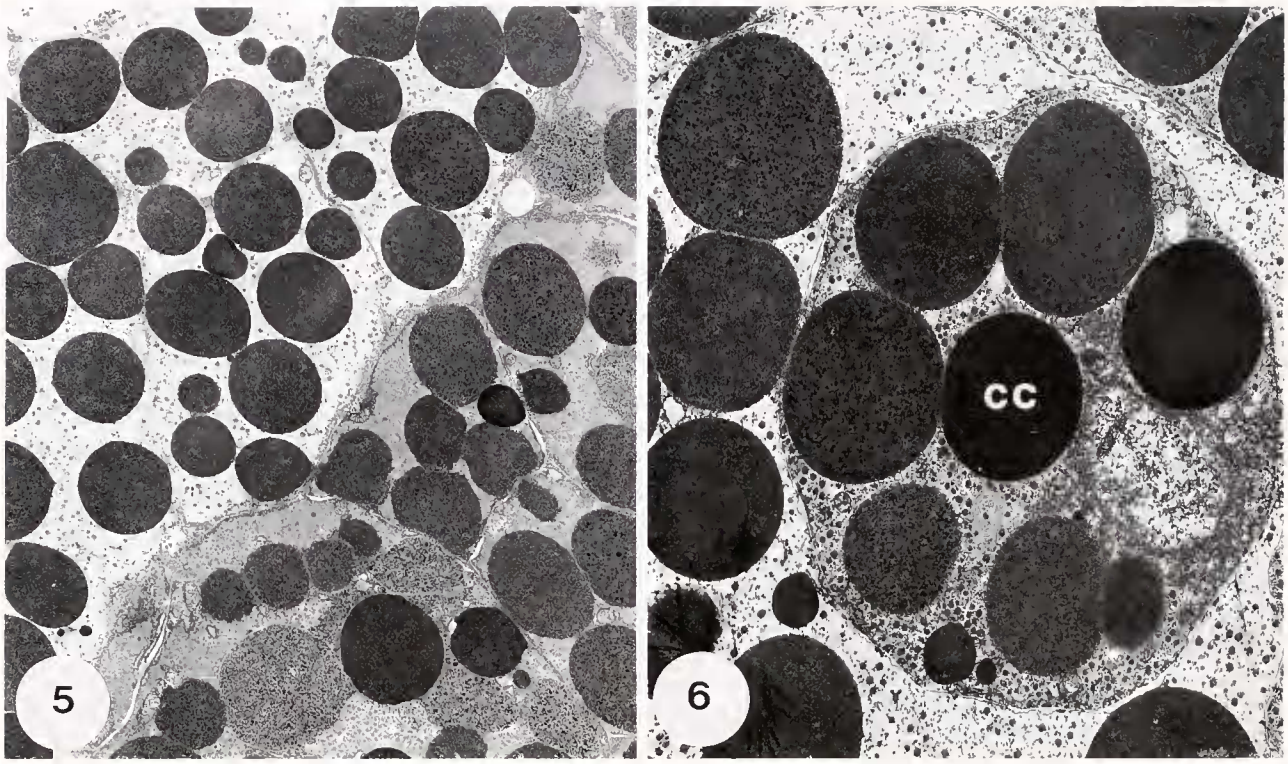
Figures 3 and 4. Transmission electron micrographs of sectioned *Molgula retortiformis* gastrulae showing the outer epidermal cells (ep) containing less yolk and glycogen than the large, centrally located cells (cc). × 3300 in Fig. 3; × 4900 in Fig. 4.

were accompanied by changes in the overall shape of the egg.

The early cleavage patterns exhibited by *M. retortiformis* embryos appeared similar to those exhibited by other ascidian embryos. The first cleavage plane bissected the narrow belt of ectoplasm into two equal regions. The two equal-sized blastomeres of a two-celled embryo continued cell division and formed a gastrula. Cells in the vegetal pole region invaginated in a manner similar to that seen in *Boltenia villosa* gastrulae. As a result of these vegetal cell movements, an archenteron resembling that of *B. villosa* formed. The ultrastructural features of the various cells that constitute a *M. retortiformis* gastrula are shown in Figures 3 and 4. The cytoplasm of the large, centrally located cells was packed with yolk and glycogen. Ectodermal cells contained less yolk and glycogen than these central cells. Other cell types, based on distinct ultrastructural features, were not evident.

Tail development was completely absent in *M. retortiformis*. No indication of a shape change of the posterior region or of notochord elongation was observed. Ampulla outgrowth was always triggered at a fixed time in development, immediately before hatching. Each juvenile developed a maximum of eight ampullae. Rhythmic contraction waves were evident in each ampulla by day 4 of development. Blood cells were evident within each ampullar lumen.

Figures 5 and 6 show the ultrastructural features of various cell types constituting day 2.5 juveniles. Yolk and glycogen stored in the egg cytoplasm persisted through day 2.5 of development and were not partitioned into any particular cell type, but were present in varying amounts in all cells. Epidermal cells contained fewer yolk granules and glycogen than the larger, central cells of a juvenile. Given that *M. retortiformis* juveniles do not start feeding until after one week of development, the energy required for all of the morphogenetic processes is likely derived from the large, yolky cells. These cells probably make up part of the adult rudiment. In striking contrast to species that produce planktonic larvae, in *M. retortiformis* juveniles have no differentiated muscle cells (compare Figs. 5 and 6 and Fig. 7). I tested the possibility that despite the absence of differentiated muscle cells, these juveniles might be able to express AChE activity. AChE histochemistry was performed on newly hatched *M. retortiformis* juveniles at day 2 of development and on day-2 larvae produced by *Halocynthia pyriformis*, *Boltenia echinata*, or *Ciona intestinalis*. The results of these experiments are shown in Figures 8 through 11 and Table I. Larvae from all three species that have indirect development showed AChE activity in tail muscle cells (Fig. 9), whereas *M. retortiformis* juveniles did not express AChE activity (Fig. 11). One hundred and sixty-three *M. retortiformis* juveniles from eight egg clutches collected during four sum-



Figures 5 and 6. Transmission electron micrographs of sectioned day 2.5 *Molgula retortiformis* juveniles. Yolk and glycogen were the predominant cytoplasmic feature of juvenile cells. Centrally located cells (cc) contain large quantities of yolk and glycogen. Differentiated muscle cells were not observed in *M. retortiformis* sections. × 3300 in Fig. 5; × 4900 in Fig. 6.

mers were tested. *M. retortiformis* juveniles lack not only larval muscle cells, but also the sensory structures present in the head region of tadpole larvae. NN18, a monoclonal antibody raised to vertebrate neurofilament protein, stained the cortical region of *B. villosa* eggs (Fig. 8). In contrast, NN18 did not stain the cortical cytoplasm of *M. retortiformis* eggs (Fig. 10). More than 100 sectioned eggs from different clutches were examined together with sectioned *B. villosa* eggs.

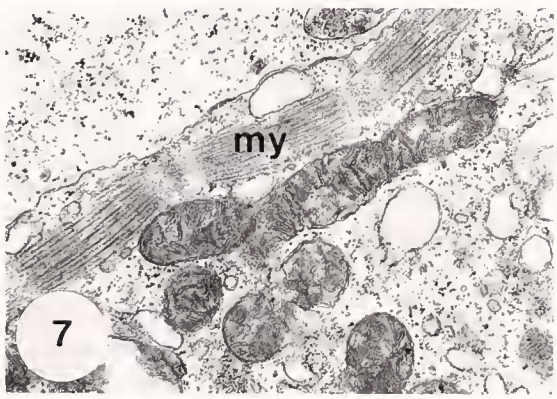
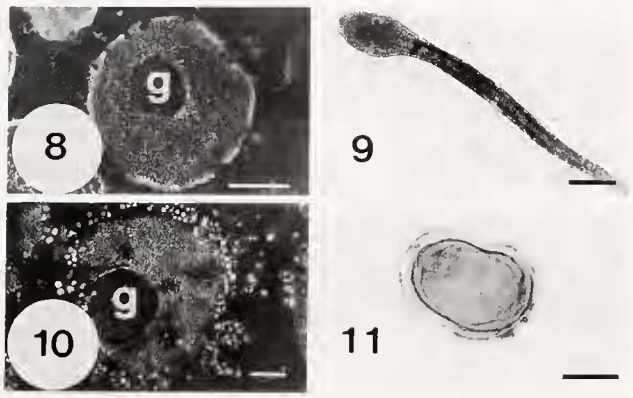


Figure 7. Transmission electron micrograph of a sectioned *Boltenia villosa* larva. Differentiated muscle cells were evident in *B. villosa* larvae, in contrast to *M. retortiformis* preparations that lacked differentiated muscle cells. my—striated myofibril. × 19,000.

Discussion

In summary, this report (1) provides new information on the ultrastructural features of *M. retortiformis* eggs, gastrulae, and day-2.5 juveniles; (2) presents ultrastructural and histochemical evidence suggesting that *M. retortiformis* embryos do not produce differentiated larval muscle cells; (3) furnishes immunocytochemical evidence that *M. retortiformis* eggs lack a cortical protein that is recognized by NN18 antibody; and (4) suggests that Berrill's substrate hypothesis is in need of revision, because *M. retortiformis* adults live on a hard, nonuniform substrate.

Large quantities of yolk and glycogen were present in the cytoplasm of eggs and most cells constituting gastrulae and day-2.5 juveniles. Two other direct-developing ascidians, *Molgula pacifica* (Bates and Mallett, 1991a,b) and *Molgula occulta* (Jeffery and Swalla, 1990), produce eggs containing large quantities of yolk and glycogen. In all three of these direct-developing molgulids, as in ascidians having indirect development (Berrill, 1975; Cloney, 1982), feeding does not begin until after the development of adult organs. Large quantities of yolk present in the cytoplasm



Figures 8–11. NN18 antibody staining of *Boltenia villosa* and *Molgula retortiformis* eggs and AChE expressions of *B. villosa* larvae and *M. retortiformis* juveniles. The cortical region of *B. villosa* eggs was stained with NN18 antibody (Fig. 8), whereas the cortical region of *M. retortiformis* eggs did not stain with NN18 antibody (Fig. 10). *M. retortiformis* follicle cells are autofluorescent. g—germinal vesicle. Fig. 9: Dark-stained AChE positive muscle cells in the tail of a *B. villosa* larva. Fig. 11: *M. retortiformis* juvenile exhibiting no AChE activity. Scale bars equal 50 μm in (8); 100 μm in (9); 50 μm in (10); 100 μm in (11).

of meroblastic types of eggs, such as those produced by birds and reptiles, directly affect patterns of cell division and modify cell movements associated with gastrulation. The presence of a few test cells within the perivitelline space of *M. retortiformis* eggs was surprising because such cells are thought to be involved in the development of a larval tail fin (Cloney, 1982). Despite the yolky cytoplasm of *M. retortiformis* eggs, early cell divisions were holoblastic, and gastrulation was similar to that in indirect-developing embryos containing less yolk. Vegetal pole cells invaginated to form an archenteron. In contrast, gastrulation in *M. pacifica* embryos is highly modified (Bates and Mallett, 1991a) and a typical archenteron never develops. Instead, the large, yolky endoderm cells within the central region of the embryo appear to physically impede the inward movements of vegetal pole cells.

Ooplasmic segregation movements and early cleavage patterns in *M. retortiformis* are similar to those in eggs and embryos that have indirect development (Conklin, 1905; Bates and Jeffery, 1988). Unlike the eggs produced by several species of *Styela* and by *Boltenia villosa*, the eggs of *M. retortiformis* do not contain colored pigment granules associated with the cortical region. However, the postfertilization movements of the egg cytoplasm of *M. retortiformis* could be studied in live eggs due to the presence of an opaque cytoplasm presumably derived from the contents of the germinal vesicle, as in other ascidians (Conklin, 1905). Opaque cytoplasm first accumulated in one region of the egg and was subsequently moved into the equatorial region where it spread out and formed a narrow cytoplasmic region. These cytoplasmic movements that have been described in the fertilized eggs of indirect-developing ascidians are thought to be important in the specification of cell fates and axial development (Conklin, 1905; Bates and Jeffery, 1988). It appears that in *M. pacifica* (Bates and Mallett, 1991a) and *M. retortiformis*, these precise movements of egg cytoplasm have been evolutionarily conserved.

The absence of myofilaments and AChE activity in *M. retortiformis* juveniles suggests that the developmental program responsible for the specification of larval muscle cells was eliminated. Myofilaments and AChE activity were also absent in *M. pacifica* juveniles (Bates and Mallett, 1991b). But at least two other molgulids that have direct development can express low levels of AChE activity (Whittaker, 1979; Jeffery and Swalla, 1990; Bates and Mallett, 1991b). The interpretation that AChE activity in a direct-developing ascidian is a vestige of larval muscle cell expression is based on Berrill's assumption that direct development evolved from species that have indirect development (1931). This assumption is being tested in several laboratories by comparing ascidian gene sequences. DNA sequence comparisons may suggest that *M. retortiformis* is most closely related to another molgulid that has direct development or to a molgulid with indirect development. Maybe *M. retortiformis* is closely related to *Molgula citrina*, an indirect-developing species that lives on the same underwater hill as *M. retortiformis*.

The elimination of differentiated muscle cells in *M. retortiformis* may be due to an evolutionary modification of the egg cytoskeleton, an idea first suggested by Swalla *et al.* (1991) in their study of direct-developing *M. occulta* embryos. NN18, an antibody raised to vertebrate neurofilament protein, stains the cortical myoplasmic region of *B. villosa* eggs, but did not stain *M. retortiformis* eggs. This result suggests that a cytoplasmic factor recognized by NN18 antibody, absent in *M. retortiformis* eggs, may be involved in larval muscle cell specification. The question of whether the antigens recognized by NN18 antibody are attached to the myoplasmic cytoskeletal domain, an egg cytoplasmic region thought to be involved in muscle cell specification (Jeffery and Meier, 1983), must await future studies.

Table I

The failure of newly hatched, day 2 Molgula retortiformis *juveniles to express acetylcholinesterase activity*

Species	Number tested	Number positive
Molgula retortiformis (D)	163	0
Halocynthia pyriformis (I)	65	56
Boltenia echinata (I)	78	77
Ciona intestinalis (I)	8	8

D—species with direct development; I—species with indirect development. Tested at day 2 of development.

Data collected from field sites in the Atlantic and Pacific oceans, on adults of *M. retortiformis* (present study) and *M. pacifica* (Bates and Mallett, 1991a) respectively, appear to conflict with Berrill's substrate hypothesis (1931). Berrill based his hypothesis on field and developmental studies of *Molgula occulta* (a direct developer) and *Molgula oculata* (an indirect developer), species that live on the sandflats along the coast of Brittany. The occurrence of *M. occulta* was attributed to habitat uniformity. Berrill suggested that tadpole development was eliminated from the life cycle because tadpoles capable of selecting a habitat are unnecessary in a uniform environment. But the largest populations of *M. retortiformis* adults live in a rocky, nonuniform habitat. Seven summers of field collections along the west coast of Vancouver Island near Bamfield Marine Station indicate that *M. pacifica* adults thrive on rocky, nonuniform habitats (Young *et al.*, 1988; Bates and Mallett, 1991a; Bates, 1993). The evolution of morphogenetic processes in ascidians has been discussed at length elsewhere (Bates, 1993, 1994), with the suggestion that the evolution of a fixed timing mechanism for triggering a rapid deployment of ampullae may be important to the reproductive success of direct-developing ascidians. The finding described in the present report that ampulla morphogenesis occurs at a fixed time in *M. retortiformis* development supports this idea.

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